Quantitative Analysis of the Mono- and Sesquiterpenoids of Douglas-fir Sapwood by Solvent Extraction and Gas Chromatography with Mass Selective Detection

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Summary

In order to investigate the relationship between terpenoid content and black bear foraging preference, an analytical method was required to quantify mono- and sesquiterpenoids present in Douglas-fir sapwood. Sapwood samples were scraped from trees, immediately frozen in liquid nitrogen, and then homogenized. A simple extraction requiring no clean-up step was performed with ethyl acetate. Extracts were analyzed by gas chromatography with mass selective detection versus external standards. The recoveries of 22 terpenoids from fortified controls were approximately 90% with good precision (relative standard deviations of approximately 10%).

1 Introduction

Published methods for the determination of terpenoids in Douglas-fir tissues have primarily focused on the composition of the needles. These studies have typically employed simultaneous steam distillation – solvent extraction with gas chromatography/flame ionization detection (GC/FID) to measure abundances of terpenoids [1]. Because of the correlation of number of carbons and FID response, paraffins have also been used as internal standards to quantify terpenoids in Douglas-fir needle extracts [2]. However, because flame ionization detection does not provide any structural information, off-line spectrometric methods are required for compound identification.

We required a simple analytical method which provided sensitive detection and identification of mono- and sesquiterpenoids in sapwood. Proper identification and quantification of the terpenoids in Douglas-fir sapwood is central to determining the roles they play in black bear (*Ursus americanus*) forage selection. Damage to Douglas-fir (*Pseudotsuga menziesii*) by foraging bears constitutes a significant loss of trees in Pacific northwest forests. An individual bear can cause extensive damage, peeling the trunks of 50 to 70 trees per day to feed on the sapwood [3]. Bears feed on the sapwood by removing the bark with their claws and scraping the sapwood away from the heartwood with their incisors. Sapwood foraging usually occurs in the spring, presumably due to the chemical composition of spring sapwood [4].

Our investigation of the terpenoids present in Douglas-fir sapwood was made possible through the development of a simple solvent extraction procedure which did not require a clean-up step. Analysis was achieved by gas chromatography/mass selective detection (GC/MSD). However, as opposed to FID, the response factors obtained from the MSD varied. Therefore, external standards of each compound identified were employed for quantitative analysis.

2 Materials and Methods

2.1 Equipment

A Hewlett-Packard Model 5890 Series II gas chromatograph equipped with electronic pressure control and a mass selective detector (MSD) (Hewlett-Packard Co., Avondale, PA) was used for terpenoid analyses. A horizontal mechanical shaker (Eberbach, Ann Arbor, MI) and bench-top centrifuge (Fisher Sci., Pittsburgh, PA) were used in the preparation of sample extracts.

2.2 Chemicals

(1*R*)-(+)-α-Pinene, (+)-camphene, (1*S*)-(–)-β-pinene, myrcene, 3-carene, α-terpinene, *p*-cymene, (R)-(+)-limonene, γ-terpinene, (±)-linalool, ((1*S*)-endo)-(–)-borneol, terpinen-4-ol, α-terpineol, (*R*)-(+)-citronellal, (*R*)-(+)-citronellol, (–)-bornyl acetate, and (+)-longifolene were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). α-Thujene was obtained from Indofine Chemical Company (Somerville, NJ). (+)-Sabinene was obtained from Fluka Chemica-BioChemika (Ronkonkoma, NY) and terpinolene from TCI America (Portland, OR). (–)-*trans*-Caryophyllene and α-humulene were purchased from Sigma Chemical Company (St. Louis, MO).

2.3 Working Standard Solutions

Since the individual reference standards were not 100% pure, the contaminants present were frequently other terpenoids of interest. Therefore, four separate concentrated standard solutions (approximately 1 mg/mL of each terpenoid) were prepared in ethyl acetate in combinations which eliminated contributions from impurities present in other standards. The four standard solutions that achieved this were: Standard Solution A: myrcene, *p*-cymene, terpinolene, citronellal, citronellol, and α-humulene; Standard Solution B: 3-carene, γ-terpinene, linalool, borneol, and bornyl acetate; Standard Solution C: α-thujene, camphene, sabinene, α-terpinene, α-terpineol, and longifolene; and Standard Solution D: α-pinene, β-pinene, limonene, terpinen-4-ol, and

caryophyllene. Three working standards were prepared in ethyl acetate from each of the concentrated standard solutions to produce individual terpenoid concentrations of approximately 1.0, 10, and 50 μg/mL. The exception to this was α-pinene which had a concentrated standard concentration of 9.77 mg/mL and resulting working standard concentrations of 9.76, 96.7, and 465 μg/mL.

2.4 Response Linearity

Response linearity of each compound was assessed by preparing five calibration solutions from each of the four concentrated standard solutions. The concentrations of the terpenoids were in the same range as the working standards. Each solution was injected into the GC/MSD and the response data subjected to linear regression analysis.

2.5 Sample Collection

Two 40 × 10 cm patches of bark were removed and the sapwood (phloem tissue and xylem oleoresin located immediately below the cork cambium) was scraped into a plastic bag. Samples were obtained at a height of 1.5 m. The freezer bag and contents were immediately placed in liquid nitrogen for approximately two minutes. After complete freezing, the samples were kept on dry ice until placed in a laboratory freezer at –24 °C. Samples were maintained frozen and homogenized with a rubber mallet.

2.6 Study Sites

Sapwood samples were obtained from three sites in western Washington. The first site (Oakville, WA) not only served as a study site, but also produced the samples used to evaluate the analytical methodology. Twenty trees were sampled for terpenoid analysis. Ten of these were sampled in duplicate to evaluate the methodology. The stand density at the Oakville Site was 160 stems/acre and the trees were 19 years old. Thirty Douglas-fir trees were sampled in each of the other two sites for terpenoid analysis only. The majority of trees at the second site (McCleary, WA) were Douglas-fir, but some western hemlock (*Tsuga heterophylla*) were also present. The stand consisted of 20 year old trees at a density of 420 stems/acre. The third site (Kelso, WA) was a Douglas-fir monoculture. The trees in this 190 stems/acre stand were also 20 years old. None of the three sites had been fertilized.

2.7 Method Evaluation

To assess the sample handling procedures employed in the field and laboratory, the ten duplicate samples obtained from the Oakville Site were each fortified in the field with 998 μg of borneol. The fortified samples were analyzed and the borneol recovery was determined.

The terpenoid recovery afforded by the extraction procedure was also evaluated. To accomplish this, a 150 g composite sapwood sample was first lyophilized to remove the majority of the volatile terpenoids and then rehydrated to the original water content. Control samples (3 – 4 g) were then fortified with 22 different terpenoids by spiking with one of four different terpenoid solutions made in acetone. The four spiking solutions were prepared in the same combinations as the concentrated standard solutions. This avoided contributions from contaminants which would lead to artificially high recoveries. Recovery of each analyte was assessed with seven replicate fortifications. The samples were analyzed and the recoveries of each terpenoid were determined.

2.8 Terpenoid Analysis

Approximately 3 – 4 g of frozen sapwood was extracted with 10.0 mL of ethyl acetate in 50-mL glass screw-top centrifuge tubes. The sapwood obtained from each tree was analyzed in triplicate. The extractions were performed on a mechanical horizontal shaker for 10 min followed by centrifugation at approximately 2500 rpm for 15 min. Extracts were injected directly into the GC/MSD without clean-up. Single injections of each of the 12 working standard solutions were also made. Terpenoids identified by their spectra and retention times were quantified versus the external standards.

The gas chromatograph was equipped with a 30 m × 0.25 mm, 5% phenyl – 95% poly(dimethylsiloxane) fused silica capillary column with a 0.25 μm film thickness (DB-5, J&W Scientific, Folsom, CA). At injection, the oven temperature program initiated at 35 °C; ramped at 5 °/min to 185 °C; and was followed immediately by a 20 °/min ramp to 300 °C, which was maintained for 9.25 min. The injection port temperature was 200 °C and the transfer line to the MSD was maintained at 280 °C. One μL splitless injections (1.00 min purge time) were made with electronic pressure control maintaining constant helium flow (1.1 mL/min). Mass spectral data were acquired from 40 to 300 *m/z*. The solvent delay was 5.0 min.

3 Results and Discussion

3.1 Response Linearity

Linear responses were observed for all compounds over the ranges of interest. For several terpenoids, the responses were not directly proportional to concentration (*i.e.* the response factors were not constant over the entire range), so three point working standard curves were employed for quantitative analysis. The coefficient of determination, slope, and *y*-intercept were determined for each compound (**Table 1**). The null hypothesis of *y*-intercept equal to zero was also tested.

3.2 Method Evaluation

Excellent recovery of borneol was obtained from the sapwood samples fortified in the field. Mean borneol recovery was 89.0% and the relative standard deviation was 10% (n = 10). Because the mass of sample varied from tree to tree, the borneol concentration resulting from fortification ranged from 13 to 22 μg/g. Borneol was not present in detectable quantities in the samples which were not fortified. These results demonstrate excellent sample integrity and analyte recovery.

The recoveries of the 22 terpenoids from the control (lyophilized) sapwood were equally good. **Table 2** provides the terpenoid concentration of the control sapwood, the target fortification level, mean recovery, and RSD (n = 7). The fortification levels were chosen to be similar to the values determined in Douglas-fir sapwood. Furthermore, the fortification levels far exceeded the inherent terpenoid concentrations of the control. Recovery was assessed by subtracting the terpenoid concentration contributed by the control.

Table 1. Linear regression analysis results.

Compound	R^2	Slope	y-intercept	p[a)]	Concentration range (μg/mL)
Myrcene	0.9998	61.10	–40.66	0.0296	0.921 to 43.9
p-Cymene	0.9998	94.88	7.36	0.680	0.945 to 45.0
Terpinolene	0.9999	78.09	–10.92	0.354	0.959 to 45.7
Citronellal	0.9997	60.49	–41.32	0.0621	0.946 to 45.1
Citronellol	0.9998	69.79	–182	0.0116	1.03 to 49.0
α-Humulene	0.9999	105	–67.08	0.0344	1.08 to 51.4
3-Carene	0.9999	75.28	–13.50	0.323	0.975 to 46.5
γ-Terpinene	0.9999	89.49	–12.76	0.320	0.962 to 45.8
Linalool	0.9996	71.79	–68.51	0.0362	0.953 to 45.4
Borneol	0.9991	87.69	–72.19	0.131	0.951 to 46.3
Bornyl acetate	0.9998	86.17	–22.58	0.266	1.06 to 50.5
α-Thujene	0.9991	112	7.80	0.167	0.971 to 43.3
Camphene	0.9999	77.54	15.39	0.332	1.03 to 49.0
Sabinene	0.9999	80.82	–11.32	0.308	0.919 to 43.8
α-Terpinene	1.000	78.36	–1.73	0.816	0.912 to 43.5
α-Terpineol	0.9999	80.93	–51.08	0.0426	1.17 to 55.7
Longifolene	0.9998	101.2	–7.46	0.755	1.24 to 59.0
α-Pinene	0.9917	58.74	1190	0.207	9.76 to 465
β-Pinene	0.9999	76.39	–0.670	0.956	0.952 to 45.4
Limonene	0.9998	86.35	3.03	0.864	1.06 to 50.5
Terpinen-4-ol	0.9998	80.19	–31.71	0.181	1.15 to 54.8
Caryophyllene	0.9999	97.95	–53.88	0.0299	1.17 to 55.7

[a)] For HO: y-intercept = 0

Table 2. Terpenoid recovery from control sapwood.

Compound	Analyte inherent to control (μg/g)	Target spike level (μg/g)	Mean % recovery	*RSD*	MLOD (μg/g)
Myrcene	ND [a)]	4.53	88.1	14	0.73
p-Cymene	0.245	2.88	104	23	–
Terpinolene	0.729	5.76	87.8	8.7	–
Citronellal	ND	5.95	95.3	5.8	1.0
Citronellol	ND	13.3	117	5.9	1.5
α-Humulene	ND	4.27	106	5.3	0.44
3-Carene	0.653	5.23	84.4	13	–
γ-Terpinene	0.424	2.88	83.8	13	–
Linalool	1.33	6.53	82.4	15	–
Borneol	ND	13.3	102	7.5	0.87
Bornyl acetate	1.51	4.21	78.7	11	–
α-Thujene	0.732	2.85	76.3	7.5	–
Camphene	0.968	6.40	86.9	18	–
Sabinene	1.88	8.08	74.2	9.8	–
α-Terpinene	0.175	2.20	93.3	14	–
α-Terpineol	1.02	6.29	94.9	7.6	–
Longifolene	0.981	3.39	93.3	9.8	–
α-Pinene	32.7	265	85.2	9.2	–
β-Pinene	3.24	27.2	84.0	7.9	–
Limonene	0.546	7.09	88.5	7.2	–
Terpinen-4-ol	1.52	4.37	76.2	8.7	–
Caryophyllene	ND	4.56	106	3.5	0.33

[a)] Not detected

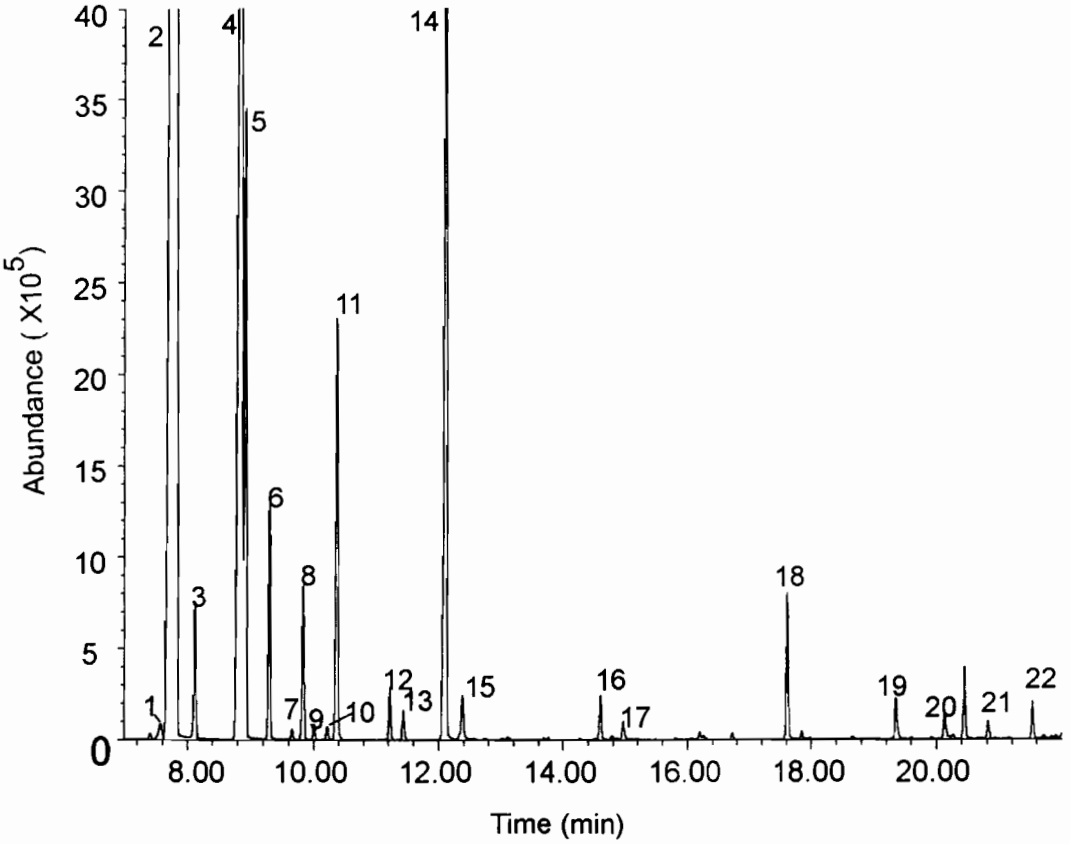

Figure 1. Gas chromatogram of a Douglas-fir sapwood extract: 1, α-thujene; 2, α-pinene; 3, camphene; 4, sabinene; 5,β-pinene; 6, myrcene; 7, α-phellandrene; 8, 3-carene; 9, α-terpinene; 10, *p*-cymene; 11, limonene; 12, γ-terpinene; 13, sabinene hydrate; 14, terpinolene; 15, linalool; 16, terpinen-4-ol; 17, α-terpineol; 18, bornyl acetate; 19, citronellyl acetate; 20, geranyl acetate; 21, longifolene; 22, α-humulene.

Table 3. Mean terpenoid concentration in Douglas-fir sapwood (μg/g and relative standard deviation).

Compound	Oakville Site (n = 20)	McCleary Site (n = 30)	Kelso Site (n = 30)
Myrcene	4.38 (56.2%)	13.6 (216%)	4.18 (51.4%)
p-Cymene	0.605 (167%)	1.00 (144%)	0.440 (132%)
Terpinolene	4.16 (70.0%)	28.7 (292%)	4.23 (66.9%)
α-Humulene	ND[a)]	0.359 (254%)	0.199 (317%)
3-Carene	6.78 (87.6%)	20.9 (133%)	7.39 (100%)
γ-Terpinene	0.534 (181%)	1.60 (208%)	0.422 (119%)
Linalool	0.400 (282%)	3.40 (260%)	0.105 (306%)
Bornyl acetate	2.25 (59.6%)	7.02 (207%)	2.22 (69.4%)
α-Thujene	0.997 (125%)	1.70 (163%)	0.722 (103%)
Camphene	2.65 (48.3%)	6.92 (224%)	2.22 (65.8%)
Sabinene	9.50 (60.8%)	64.3 (288%)	11.1 (80.9%)
α-Terpinene	0.310 (195%)	0.857 (169%)	0.264 (170%)
α-Terpineol	0.462 (266%)	1.21 (237%)	0.0755 (547%)
Longifolene	1.24 (135%)	3.22 (332%)	0.889 (139%)
α-Pinene	229 (59.4%)	477 (164%)	229 (69.0%)
β-Pinene	18.3 (57.9%)	47.3 (194%)	18.9 (58.2%)
Limonene	7.43 (117%)	22.1 (211%)	9.28 (75.6%)
Terpinen-4-ol	1.05 (215%)	1.67 (235%)	0.442 (191%)
Caryophyllene	0.417 (264%)	0.532 (261%)	0.576 (260%)

[a)]Not detected

The method limit of detection (MLOD) was also evaluated for those terpenoids which were completely absent in the control sapwood. MLOD was defined as the concentration of analyte required to produce a signal equal to three times the chromatographic noise measured peak-to-peak. MLODs were determined for myrcene, citronellal, borneol, citronellol, caryophyllene, and α-humulene only, since these compounds were not present in detectable quantities in the control sapwood (Table 2). Fortifying blank samples with a known amount of analyte provides the best assessment of MLOD because the effect of analyte recovery on detection is intrinsically considered. Recovery of all terpenoids would be expected to be similar to those found for these monoterpenes (myrcene), oxygenated monoterpenes (citronellal, citronellol, and borneol), and sesquiterpenes (caryophyllene, and α-humulene).

3.3 Terpenoids Present in Douglas-fir Sapwood

The major terpenoids quantified in Douglas-fir sapwood were: α-pinene, β-pinene, sabinene, limonene, 3-carene, myrcene, camphene, and terpinolene (in order of abundance). **Figure 1** shows a typical chromatogram obtained from the chromatographic analysis of sapwood extracts. The mean terpenoid concentrations and relative standard deviations (RSDs) determined in Douglas-fir sapwood are provided in **Table 3** for each site. While there was considerable variation in terpenoid concentration within a site, analyte recovery results indicate that the analytical methodology was not the source of variation.

Citronellyl acetate, α-phellandrene, sabinene hydrate, fenchyl alcohol, and geranyl acetate have also been tentatively identified by their spectra and anticipated retention times (based on boiling point). To our knowledge, longifolene, α-thujene, and α-phellandrene have not been previously reported in Douglas-fir tissues. However, we did not detect citronellal, citronellol, farnesyl acetate, 1,8-cineole, *cis*-ocimene, or β-phellandrene which have been reported in Douglas-fir needles [1,2].

Terpenoid allocation is thought to be under environmental and genetic control. Each species or population has its own distinctive terpenoid profile [5]. These profiles may then be affected by numerous environmental controls. While allocation of secondary metabolites is probably not effected by carbon supply [6], increases in light intensity have been shown to result in elevated terpenoid concentrations in woody plants [7,8]. Nitrogen fertilization has had a negative effect [7] or no effect [11] on terpenoid allocation. Inductive changes to terpenoid concentrations due to stress and/or herbivory are also well known [5,10].

Development of this simple quantitative method allowed for the investigation of the role of terpenoids in black bear sapwood foraging. Concentration data generated by chemical analyses indicated that terpenoids have a deterrent effect on black bear sapwood preference [11].

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Retention Properties of a Cyanopropylsiloxane-Bonded Silica-Based Sorbent for Solid-Phase Extraction

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Summary

The characteristic kinetic and retention properties of a silica-based cyanopropylsiloxane-bonded sorbent for solid-phase extraction are described. Abraham's solvation parameter model is used to characterize the contribution of individual intermolecular interactions to retention under liquid chromatographic and sample processing conditions with aqueous methanol mixtures as the mobile phase. The main features governing retention by the sorbent are the solute's size and hydrogen-bond basicity; interactions of a dipole type are not significant when aqueous methanol solutions are employed as the mobile phase. Compared to typical silica-based octadecylsiloxane-bonded sorbents the greater difficulty of forming a cavity in the solvated cyanopropylsiloxane-bonded sorbent more than offsets the more favorable dipole-type and solute hydrogen-bond base interactions of the cyanopropylsiloxane-bonded sorbent. It is shown that there are no practical circumstances for which a cyanopropylsiloxane-bonded sorbent would be more useful than a typical ODS sorbent for the isolation of organic non-electrolytes from water by solid-phase extraction.

1 Introduction

Solid-phase extraction is widely used to isolate and concentrate organic contaminants from water [1, 2]. It has largely replaced liquid-liquid extraction for this purpose and is the basis of a number of "official" or "regulatory" methods world wide. Solid-phase extraction has traditionally been performed using cartridge devices and more recently using particle-loaded membranes and particle-embedded glass fiber discs. The capacity of these devices to retain analytes is a function of their kinetic and retention properties [3–6]. The breakthrough volume for a sampling device depends mainly on the amount of sorbent and its characteristic retention properties; the breakthrough volume is only weakly dependent on the efficiency of the devices if a certain minimum efficiency is achieved. The retention properties of a sorbent can be characterized using Abraham's solvation parameter model expressed in the following form [4]

$$SP = \frac{mV_X}{100} + r R_2 + s \pi_2^H + a \alpha_2^H + b \beta_2^H \quad (1)$$

where SP is a free energy related solute property, V_X is the solute's characteristic volume, $s\pi_2^H$ is a measure of the solute's ability to stabilize a neighboring dipole by virtue of its capacity for orientation and induction interactions, R_2 is the solute's excess molar refraction, and α_2^H and β_2^H are parameters characterizing the solute's effective hydrogen-bond acidity and hydrogen-bond basicity, respectively. The system constants *m, r, s, a, b,* and *c* are ideally independent of a solute's identity and are characteristic of the sampling system, consisting of the solvated stationary phase and sample solvent. The system constants are obtained by multiple linear regression analysis by determining the property *SP* for a series of solutes with known explanatory variables. System constants have been determined for octadecylsiloxane-bonded silica particle-loaded membranes [4, 7], particle-embedded glass fiber discs [8], and cartridges [5] using either capacity factor values determined by forced flow thin layer or high pressure liquid chromatography, or from the determination of breakthrough volumes, as the free energy related solute property, *SP*, in equation (1). The breakthrough volume method was also used to determine the system constants for a porous polymer particle-loaded membrane [7, 9]. Once established, the property *SP* can be estimated for any solute in the same sampling system for which the solute explanatory variables are known or can be reasonably estimated from empirical combining rules. At present explanatory variables are available for in excess of 1000 compounds [10, 11]. To date, all the sorbent parameters available have been determined for relatively non-polar sorbents and the purpose of this paper is to provide similar data for a polar silica-based cyanopropylsiloxane-bonded sorbent so as to identify the origin of retention differences between the various sorbent types. In a related study Park *et al.* [12] used the solvatochromic parameters of Taft and co-workers to determine the retention characteristics of three cyanopropylsiloxane-bonded silica-based stationary phases used in reversed-phase liquid chromatography. Although the conclusions of that study are not necessarily at variance with expectations from the results presented here it should be noted that the explanatory variables used differ numerically and in their origins from those we have used. The explanatory variables proposed by Abraham, and used in this paper, are preferred because they are clearly thermodynamically related while the solvatochromic parameters used by Park *et al.* are not, and are based on spectroscopic measurements of indicator compounds.

2 Experimental

All solvents and water were Omnisolv grade from EM Science (Gibbstown, NJ, USA). Other chemicals were reagent grade or better and obtained from several sources. The solid-phase extraction cartridges were 6-mL disposable extraction columns from J.T. Baker (Philipsburg, NJ, USA), product number 7021-07, from lot H02571 containing cyanopropylsiloxane-bonded silica packing.